CHAPTER – 5
INVESTIGATION OF THE ANTI-INFLAMMATION AND WOUND HEALING EFFECT OF Decalepis hamiltonii AND Solanum muricatum

5.1 Introduction:

Inflammation is one of the primary defense mechanism that helps the body to protect itself against infection, allergens, toxic chemicals and burns (Vodovotz et al., 2009; Ferrero-Miliani et al., 2007). Inflammation involves complex series of events including dilation of arterioles, venules and capillaries with increased exudation of fluid and vascular permeability. Inflammation can be either acute or chronic. (Cuzzocrea, 2005). Steroidal drugs, NonSteroidal Anti-Inflammatory Drugs (NSAIDs) and immuno-suppressant drugs have been widely used in the relief of inflammatory diseases. Recently many medicines derived from plant and plant products are considered to be effective with less side effect for the treatment of various diseases including inflammation and pain (Su et al., 2011).

The skin is one of the largest organ in our body system with the resolve to help as wall against external agent. Wound is visible and are results of separate cell death or break of the skin. The wound symptoms are loss of function below the wound site, painful and swelling of tissue, bleeding, redness and pus formation (Rashed et al., 2003; Tarnawski and Halter, 1995). Wound healing is a complex and intricate process that is initiated by injury and often terminated by creation of scar
(Rubin and Fabrex, 1996). The wound healing process involves different phases such as inflammatory phase, proliferation phase and tissue remodeling (Fulzele et al., 2002). Moreover, several traditional wound care agents are available, which exhibit side effects and lacks scientific evaluation (Govindarajan et al., 2004). It has been reported that, herbal based drugs and natural products are less toxic and free from side effects when compared with synthetic drugs (Charde et al., 2006).

A maximum number of medicinal plants have been used in early days for treatment of various skin diseases. These include *Martynia annua* (Santram and Singhai 2011), *Siegesbecka pubescens* (Wang, 2011), *Mimosa pudica* (Kokane et al., 2009), *Crotalaria verrucosa* (Meena kumarai et al., 2010), *Butea monosperma* (Sumitra et al., 2005), *Centaurea iberica* (Koca et al., 2009), *Trichosanthes dioica Roxb* (Shivhare et al., 2010) and *Hypericum perforatum* (Suntar et al., 2010) which have been widely reported in Siddha, Ayurveda and Unani system of medication for the treatment and management of wounds (Kumar et al., 2007).

In this study we had to evaluated the anti-inflammatory (acute and chronic) and wound healing (excision and incision) activity of *D.hamiltonii* and *S.muricatum.*
5.2 Materials and Methods

5.2.1 Chemicals

Carrageenan, formaldehyde, hydroxyproline and cysteine were obtained from Sigma chemicals, St. Louis, USA. Gum Acacia was purchased from HiMedia, Mumbai. Papain were obtained from Sisco Research Lab, Mumbai, India. Nitrofurazone ointment were procured from Biomedica International (Punjab, India). All other chemicals used were of analytical grade.

5.2.2 Animals

Male BALB/c mice (20-25g) and Male Wistar rats (120-150g) were purchased from Small Animals Breeding Station, Veterinary College, Mannuthy, Trichur. The animals were maintained under controlled condition as explained in chapter 4. The animal experiments were conducted after getting approval from Institutional Animal Ethics Committee, Karunya University.

5.2.3 Preparation of Extract

*D. hamiltonii* root extract and *S. muricatum* fruit extract were prepared as explained in chapter 3. For *in vivo* studies *D. hamiltonii* extract and *S. muricatum* extract were resuspended separately in 1% gum acacia and injected at a
concentration of 20 mg/kg B.wt., (i.p.) and 10mg/kg B.wt., (i.p.) for ten consecutive
days.

5.2.4 Preparation of ointment

The ointment was prepared using the plant extract. The composition of the
ointment involves hard paraffin (0.5g), white soft paraffin (8.0 g), wool fat (0.5g),
ceto stearyl alcohol (0.5g) and the plant extract (5%) (Dash and Murthy 2011).

5.2.5 Experimental design for excision wound

Wistar rats were divided into five group (n=6/group). Group I: Normal
control; Group II: Wound bearing control; Group III: Wound + Nitrofurazone;
Group IV: Wound + D.hamiltonii; Group V: Wound + S.muricatum.

5.2.6 Experimental design for Incision wound

Wistar rats were divided into five group (n=6/group). Group I: Normal
control; Group II: Wound bearing control; Group III: Wound + Nitrofurazone;
Group IV: Wound + D.hamiltonii; Group V: Wound + S.muricatum.
5.2.7 Excision wound

The dorsal side of the animals were cleanly shaved using a sterile surgical blade an impression of 1x1cm² was made on the shaved dorsal region. The animals were kept under mild ether anaesthesia during this procedure (Morton and Malone, 1972: Sadaf et al., 2006). The ointment containing the plant extract was tropically administered until day 14th and the parameters such as percentage of wound contraction by using this formula: % wound contraction = [(Initial wound size – specific day wound size) / Initial wound size] X 100. Hydroxyproline (Bergman and Loxley, 1970), hexosamine (Elson and Morgan, 1933) and uronic acid (Bitter and Muir, 1962) was determined.

5.2.8 Incision wound

Para vertebral straight incision of 6 cm length was made through the entire thickness of the skin, on their side of the vertebral column with the help of the sharp scalpel. After complete haemostasis, the wounds were closed by means of interrupted sutures placed at approximately 1 cm apart. Animals were treated daily with drugs, as mentioned above under excision wound model from 0th day. The sutures were removed on 7th day. The wound tensile strength was estimated on 10th day by using Tensile strength and Elongation test apparatus (Instron 6021) (Figure 5.8, 5.9 & 5.10).
5.2.9 Assessment of anti-inflammatory activity

5.2.9.1 Carrageenan model

BALB/c animals were divided into 3 groups (n=6/group). Group I: normal untreated control; Group II: \textit{D.hamiltonii} Group III: \textit{S.muricatum} extract given through i.p., injection for 10 consecutive days. The last dose of drug was provided 60 min before induction of inflammation. Similarly, all mice received a subcutaneous injection of 0.1 ml of 1\% (w/v) Carrageenan solution in the right hind paw to induce the inflammation. The volume of paw was measured initially and then 30 min interval up to 8 hour after injection using vernear caliper.

5.2.9.2 Formaldehyde model

Another set of BALB/c animals were divided into three groups (n=6/group). Group I: normal untreated control; Group II: \textit{D.hamiltonii} Group III: \textit{S.muricatum} extract given through i.p., injection for 10 consecutive days. The last dose of drug was provided 60 min before induction of inflammation. Similarly, all mice received a subcutaneous injection of 0.1 ml 2\% (v/v) formaldehyde solution in the right hind paw to induce the inflammation. The volume of the hind paw was measured initially to obtain the baseline value before the injection; thereafter, measures of dorsal and then measured on 5 consecutive days using a vernier caliper.

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5.2.10 Statistical Analysis

For all the in vivo studies statistical analysis were performed according to the details given in chapter 4.

5.2.11 Histopathology

On 14\textsuperscript{th} day, post wounding skin tissue wound site were excised. The specimen was fixed in 10\% formaldehyde, dehydrated and embedded in paraffin, and routine 4\(\mu\)m sections were prepared. Tissues were stained with eosin and hematoxylin. The images were captured at 10X magnification.

5.3 Results

5.3.1 Carrageenan and formaldehyde induced paw inflammation

The effect of \textit{D.hamiltonii} and \textit{S.muricatum} on carrageenan and formaldehyde induced paw inflammation is shown in figure 5.1 and 5.2 respectively. Treatment with the extract showed marked reduction in the paw inflammation compared with control animals. The paw edema of control animals were found to be 0.37 cm after 1h of carrageenan administration which gradually increased to a peak of 0.50 cm by 3\textsuperscript{rd} hour followed by gradual reduction to 0.22 cm by 48h. Treatment with \textit{D.hamiltonii} and \textit{S.muricatum} does not show marked
reduction in the early phase (1hr) but the paw edema was significantly decreased during the late phase. At the end of the experimental study (24h) the paw edema in *D.hamiltonii* and *S.muricatum* treated group were found to be 0.17 cm and 0.18 cm respectively Similarly *S.muricatum* treated animals also showed significant reduction in paw edema induced by formaldehyde. On day 5 the paw edema in control group were found to be 0.42 cm whereas *D.hamiltonii* and *S.muricatum* treatment could significantly reduce the paw edema to 0.29 cm and 0.31 cm respectively.
Values shown are mean (± SD) of six mice/treatment group. *p<0.05 **p<0.01 compared with control animals.

Figure 5.1: Effect of *D.hamiltonii* and *S.muricatum* on carrageenan induced-paw edema.
Values shown are mean (± SD) of six mice/treatment group. **p<0.01 compared with control animals.

Figure 5.2: Effect of *D.hamiltonii* and *S.muricatum* on formaldehyde-induced-paw edema.
5.3.2 Percentage of wound contraction

Percentage of wound contraction was assessed by tracing the wound area on transparent graph paper from which the wound surface area was estimated on 0, 2, 4, 6, 8, 10, 12, and 14th day. The wound surface area was calculated by percentage of wound contraction with initial size of the wound as 100%. On 14th day, *D.hamiltonii* and *S.muricatum* treated groups showed 99±0.5 and 97±1.1 wound contraction compared with control groups (85±2.5). The standard drug nitrofurazone also showed significantly contraction in wound (94±1.7) compared with the control group (Figure 5.4).

5.3.3 Effect of *D.hamiltonii* and *S.muricatum* on hydroxyproline content in excision wound model

The effect of *D.hamiltonii* and *S.muricatum* on hydroxyproline level shown in Figure 5.5. The hydroxyproline content was significantly (p<0.01) increased in *D.hamiltonii* (21.3±1.1) and *S.muricatum* (20.6±1.2) treated group compared with control group (12.7±1.7). The nitrofurazone treated group also increased the hydroxyproline level (17.7±0.6) compared with control animals.
Figure 5.3: Effect of *D. hamiltonii* and *S. muricatum* on wound contraction.

A: Normal skin; B - E: Day 0;

F - I: Day 7; J - M: Day 14
Value are expressed as mean ± SD, **p<0.01 compared to wound bearing control group.

Figure 5.4: Effect of *D. hamiltonii* and *S. muricatum* on percentage of wound contraction
Value are expressed as mean ± SD, *p<0.05, **p<0.01 compared to wound bearing control group.

**Figure 5.5:** Effect of *D.hamiltonii* and *S.muricatum* on hydroxyproline content in excision wound model
5.3.4 Effect of *D. hamiltonii* and *S. muricatum* on hexosamine level in excision wound model

The tissue hexosamine level of during excision wound is given in Figure 5.6. Significant (p<0.01) increase in hexosamine content were observed in *D. hamiltonii* treated group (886±22.3) and *S. muricatum* treated group (892±19.5) compared with wound bearing control animals (691±12.5).

5.3.5 Effect of *D. hamiltonii* and *S. muricatum* on uronic acid level in excision wound model

As depicted in Figure 5.7 a marked significantly (p<0.01) increase uronic acid level in *D. hamiltonii* and *S. muricatum* treated group (103.7±1.26 and 84.7±1.62 respectively) compared with control group (84.7±1.62). Treatment with standard drug nitrofurazone increased in the concentration of uronic acid content (97.5±1.52) (Figure 5.7).
Value are expressed as mean ± SD, **p<0.01 compared to wound bearing control group.

**Figure 5.6: Effect of *D.hamiltonii* and *S.muricatum* on hexosamine level in excision wound model**
Value are expressed as mean ± SD, *p<0.05, **p<0.01 compared to wound bearing control group.

Figure 5.7: Effect of *D.hamiltonii* and *S.muricatum* on uronic acid level in excision wound model
5.3.6 Effect of *D. hamiltonii* and *S. muricatum* on the skin tensile strength during incision wound model

Effect of *D. hamiltonii* and *S. muricatum* on tensile strength is shown in Figure 5.8. The tensile strength was significantly (*p*<0.01) increased in *D. hamiltonii* (465.36±21.05) and *S. muricatum* (474.36±7.9) treatment compared with control group (339.69±26.44). The nitrofurazone treated group also showed significantly (*p*<0.01) increased tensile strength to (451.31±15.23) compared with control animals.

5.3.7 Histopathological study

Histopathological examination of the excision tissues section was also used to access the effect of *D. hamiltonii* and *S. muricatum* on wound healing as presented in Figure 5.11. The *D. hamiltonii* and *S. muricatum* treated group showed deposition of collagen fibers with prominent blood vessel formation (Figure 5.11D and 5.11E) which confirms wound healing process compared with the control group (Figure 5.11B).
Value are expressed as mean ± SD, **p<0.01 compared to wound bearing control group.

**Figure 5.8: Effect of *D. hamiltonii* and *S. muricatum* on skin tensile strength**
Figure 5.9: Tensile strength and Elongation test apparatus (Instron 6021)
Figure 5.10: Steps involved in Tensile strength measurement
A: Normal skin;  
B: Wound bearing control;  
C: Wound + nitrofurazone  
D: Wound + *D. hamiltonii*;  
E: Wound + *S. muricatum*

**Figure 5.11: Histopathological analysis (40X magnification)**
5.4 Discussion:

Carrageenan when injected through subcutaneous injection into the mice paw produces plasma extravazation (Szolcanyi et al., 1998) and inflammation characterized by increased tissue water and plasma protein exudation with metabolism of arachidonic acid by both lipoxygenase enzyme and cyclooxygenase pathways followed by neutrophil extravazation (Gamache et al., 1986). The histamine and serotonin will be released in the early phase followed by prostaglandins especially bradyklinin will be released during the carrageenan-induced inflammation part (Kulkarni et al., 1986). In the light of these data, *D.hamiltonii* and *S.muricatum* extract seem to be more effective in the second phase of acute inflammation than in first phase. Therefore, *D.hamiltonii* and *S.muricatum* extract may block bradykinin and/or prostaglandin release better than serotonin and/or histamine release.

Chronic inflammation is a pathological condition characterized by concurrent active inflammation and tissue destruction. The formalin-induced paw edema closely resembles rheumatoid arthritis (Greenwald, 1991). It was also observed that *D.hamiltonii* and *S.muricatum* could significantly inhibit formaldehyde induced chronic inflammation. The results of the present study show that *D.hamiltonii* and *S.muricatum* having inhibiting activity over acute and chronic
inflammation. The mechanism could be due to the inhibition of the formation of inflammatory mediators.

Contraction of the wound was promoted on dat 1 and prolonged till day 14. *D.hamiltonii* and *S.muricatum* treatment showed faster epithelization of wound compared with the wound bearing control animal. The extracts showed similar results compared with Nitrofurazone. The extract based ointment could significantly improve the healing process as evidenced by changes in hydroxyproline, hexosamine and uronic acid levels. The skin histopathological also supports the wound healing potential of the extracts.

Hydroxyproline is an amino acid which is required synthesis of protein collagen. It has been used as an indicatory to determine collagen synthesis (Nayak et al., 2010). Collagen is integrity to the tissue matrix. It is plays an important role in homeostasis and epithelization at a later phase of wound healing. The increase in hydroxyproline content indicate that there was an enhance production of collagen and faster rate of healing wound. The process of wound healing depends on the regulated biosynthesis, new collagen deposition and successive maturation (Tang et al., 2007). Hydroxyproline level was significantly increased after *D.hamiltonii* and *S.muricatum* treatment compared with control animals.
The fibroblasts synthesize glycosaminoglycans and proteoglycans in the wound area. Glycosaminoglycans are made up of repeating disaccharide containing hexosamine and hexuronic acid (Sumitra et al., 2005). The extracellular matrix to be synthesized the first compound during wound healing (Nithya et al., 2003). Hexosamine, the ground substratum for collagen synthesis, is known to increase during early stage of wound healing, and decrease in the later stage. The higher amount reflects an enhanced synthesis of glyconaminoglycans. The hexosamine level was increased redirect the maintaining the collagen molecules by enhancing ionic and electrostatic interactions (Nayak et al., 1999) in the *D.hamiltonii* treated groups as well as *S.muricatum* treated group.

The elevation of wound healing activity is also proved by an increase in tensile strength after *D.hamiltonii* or *S.muricatum* treatment. Commonly collagen provides the strength to the tissues and cross linkages between collagen fibers (Lodhi et al., 2006; Madden and Peacock, 1968). The higher breaking strength indicates better healing of wound. This observation confirms that *D.hamiltonii* and *S.muricatum* possesses good wound healing property so far as tensile strength of wound healing tissue is concerned. The histopathological analysis also confirmed the wound healing efficacy of *D.hamiltonii* and *S.muricatum* as evidenced by fibroblast proliferation, epithelization collagen formation and increase in blood vessel formation.